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# Tubular antigen-associated renal disease in New Zealand white rabbits

M. J. NICOL, J. H. MILLER & T. J. NEALE* *Department of Physiology, Victoria University of Wellington, Wellington, New Zealand, and* *Department of Medicine, Wellington Clinical School of Medicine, Wellington, New Zealand*

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## SUMMARY

Rabbits immunized with autologous renal tubular antigen (Fx1A) developed tubulointerstitial nephritis whereas sheep anti-Fx1A antibody administered i.v. produced glomerulonephritis (GN). This lesion showed heavy granular glomerular deposition of immunoglobulin and subepithelial electron dense deposits, early proteinuria, leucocyte independence and a temporal pattern of quantitated glomerular antibody binding distinct from that reported to occur in passive Heymann's nephritis in rats. Isoelectric focusing followed by immunoblotting of deoxycholate-soluble Fx1A antigens with the heterologous and autologous antibodies, indicated species differences in epitope recognition which could account for dissociation between the two models.

**Keywords** tubular antigen glomerulonephritis *in situ* immune deposits

## INTRODUCTION

Rabbits immunized with a crude homologous kidney preparation do not develop active Heymann's nephritis (AHN) as seen in rats (Heymann *et al.*, 1959; Edgington, Glassock & Dixon, 1967a; Neale *et al.*, 1982; Neale & Wilson, 1982a) but extensive tubulointerstitial fibrosis, tubular degeneration, lymphocytic infiltration and granular deposition of IgG and complement (C3) along the basement membrane of the proximal convoluted tubule (TBM) (Klassen, McCluskey & Milgrom, 1971; Klassen, Milgrom & McCluskey, 1977; Unanue, Dixon & Feldman, 1966). Raising anti-rat tubular antigen antibodies in the rabbit, produced a proteinuric glomerular lesion (Barabas & Lannigan, 1981). These workers concluded that this response was not autoimmune, but subsequently have presented observations on a glomerular lesion produced by heterologous anti-rabbit 'fraction 3' antibody in rabbits (Cornish, Barabas & Lannigan, 1984).

We report here the partial characterization of a new model of experimental GN produced in New Zealand white rabbits by the *in situ* glomerular binding of heterologous anti-rabbit Fx1A globulin, and compare this with observations made in rabbits immunized with homologous Fx1A.

## MATERIALS AND METHODS

*Experimental animals.* New Zealand white rabbits (NZW) weighing 2·4–4·0 kg were used. Open renal biopsy excluded any pre-existing renal lesion (Neale, Woodroffe & Wilson 1984).

Correspondence: Dr T. J. Neale, Department of Medicine, Wellington Clinical School of Medicine, Wellington, New Zealand.

*Preparation of rabbit Fx1A.* Fx1A was prepared from NZW kidneys by a modification of the method of Edgington *et al.* (1967b). Lyophilized material was stored at −20°C.

*Preparation and characterization of anti-rabbit Fx1A globulin.* A sheep was immunized intramuscularly with 100 mg Fx1A in complete Freund's adjuvant (CFA) at 2-weekly intervals. 2 weeks following the fifth injection serum was absorbed with 1:5 normal rabbit serum in phosphate buffered saline (PBS) pH 7·2, 0·15 M and rabbit erythrocytes. Complement was inactivated at 56°C for 1 h. Globulin was obtained by twice precipitating in 50% saturated ammonium sulphate. This was resuspended in, and dialysed for 48 h against, PBS. A non-specific globulin was prepared from the serum of a normal sheep. Ouchterlony double diffusion with the specific globulin at a dilution of 1:64 produced a precipitation line with Fx1A at 1 mg/ml (in PBS). Sheep anti-rabbit glomerular basement membrane (GBM) antibodies could not be detected in the specific globulin by radioimmunoassay (Holdsworth, Wischusen & Dowling, 1983). By indirect IF microscopy the

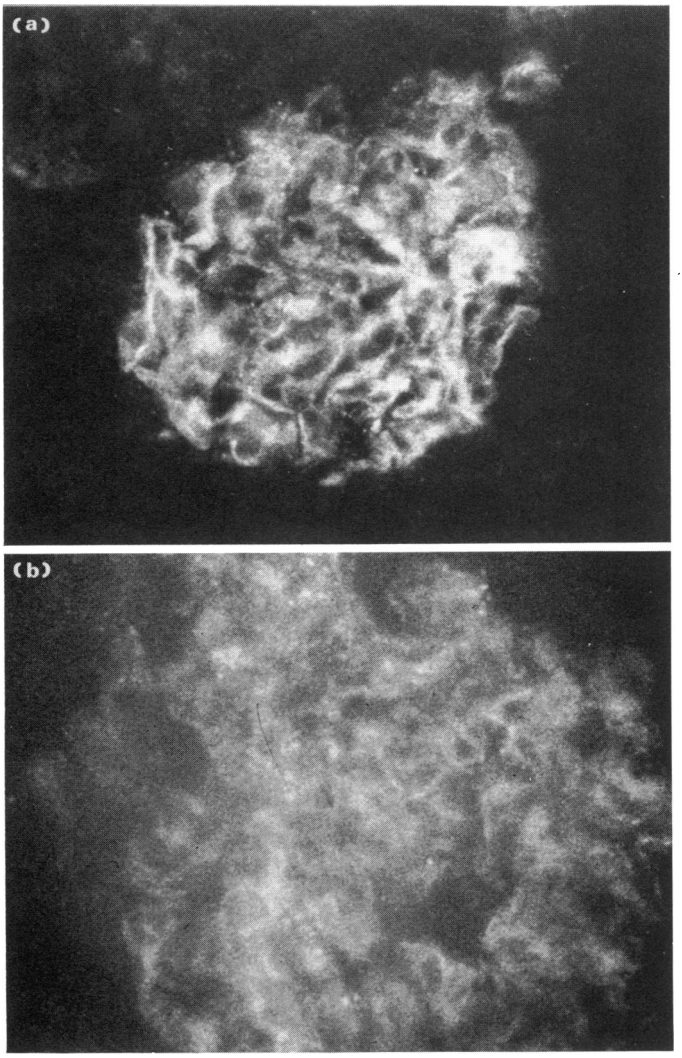


**Fig. 1.** (a) IF photomicrograph of a rabbit glomerulus stained with FITC rabbit anti-sheep IgG antibody following sheep anti-rabbit Fx1A globulin administration. The GCWs show a confluent granular deposition of sheep IgG. ×240. (b) IF photomicrograph of a rabbit glomerulus stained with FITC rabbit anti-sheep IgG antibody following sheep anti-rabbit Fx1A globulin perfusion through a bloodless kidney. ×240.

specific, but not the non-specific globulin, reacted with tubular brush border with a titre of 1:640 and weakly in a granular pattern with glomeruli in normal rabbit kidneys.

*Urine protein measurement.* Urine was obtained by transurethral catheterization prior to experimental manipulation, and at sacrifice by bladder puncture. Trichloracetic acid (TCA) precipitable protein concentration was determined by the method of Lowry *et al.* (1951).

*Light microscopy (LM).* Renal cortex was fixed in phosphate buffered formalin for 7–10 days, dehydrated in ethanol, cleared in toluene and embedded in wax. Sections (1–2 μm) were cut on a rotary microtome (Leitz 1510) and stained with periodic acid Schiff.

*IF microscopy.* Direct: Cortex was snap frozen in liquid nitrogen and stored at −80°C. 3 μm sections were stained with fluorescein isothiocyanate conjugated (FITC) rabbit anti-sheep Ig (Dako Laboratories, Glostrup, Denmark) to demonstrate heterologous antibody. Pre- and post-treatment sections (48 h onwards) were stained for autologous Ig with FITC swine anti-rabbit Ig (Dako). C3 was detected using FITC goat anti-rabbit C3. (Nordic Immunological Laboratories B.V., The Netherlands).

Indirect: Sheep anti-rabbit Fx1A globulin or the acid eluate obtained from the kidneys of Fx1A immunized rabbits (see below) was incubated as a first antibody for 30 min with normal rabbit kidney. Following three washes in PBS, FITC rabbit anti-sheep or swine anti-rabbit Ig, respectively, was applied for 30 min. Sections were mounted in 90% glycerol/10% PBS, and viewed immediately.

*Transmission Electron Microscopy (TEM).* Cortex was fixed in half strength Karnovsky fixative for 1 h, transferred to cacodylate buffered osmium tetroxide, embedded in Epon 812 and ultra-thin sections cut. Grids were viewed in a Seimens 102 transmission electron microscope following staining with lead citrate and uranyl acetate.

*The heterologous anti-Fx1A model.* Rabbits received 10 mg/kg body weight of absorbed globulin IV, and were killed at 1, 2, 12, 24 and 48 h and at 4, 7 and 14 days ($n = 3$–6). Urine was obtained for protein measurement, and renal cortex for immunohistological and ultrastructural examination. ation.

The effects of specific globulin were assessed in leucopenic rabbits (white cell count (WCC) < 1000 cells/mm$^3$) to determine the role of leucocytes in the mediation of the lesion ($n = 3$). Leucopenia was induced by IV administration of di-(2-chlorethyl) methylamine (Mustine, Boots Co. Ltd, UK), (Cochrane, Unanue and Dixon, 1965), 2 mg/kg body weight.

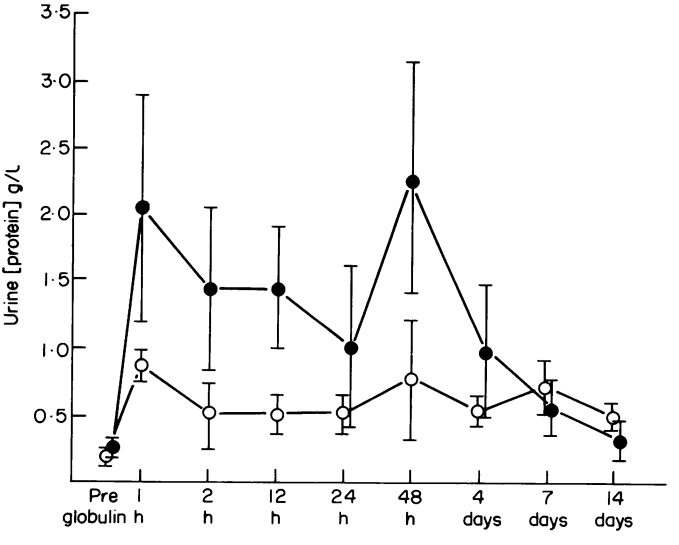


**Fig. 2.** Urinary protein excretion with time following IV sheep anti-rabbit Fx1A globulin (●) or IV normal sheep globulin (○).

Control animals received 2·5 ml of normal saline ($n=3$). Rabbits were killed at 48 h, and urine and cortex analysed.

*Iodination of globulin fractions.* For quantitative binding studies anti-Fx1A and non-specific globulins were labelled with 2 Ci of $^{125}I$ and $^{131}I$ respectively (McConahey & Dixon, 1966). Free iodine was removed by Sephadex G75 chromatography. Specific activity was determined by 10% TCA precipitation. Only globulins with >90% of the counts precipitable were used. Protein concentration of the labelled fractions was determined by the method of Goodwin & Choi (1970).

*Binding of labelled globulins.* Rabbits received simultaneously i.v. 5 mg of the labelled globulins and were sacrificed at 24 and 48 h and at 7 days. 5 ml of heparinized blood was obtained. Perfusion with PBS via the left ventricle was performed until all organs blanched. Tissue was obtained from liver, gut, spleen, lung and kidney and wet weight determined. Following homogenization in a Sorval Omnimixer and washing in PBS, specific binding was calculated from the paired label equation (Wilson *et al.*, 1971).

*Perfusion of bloodless kidneys.* To document direct binding of anti-rabbit Fx1A globulin in the absence of circulating antigen the globulins were perfused through bloodless normal rabbit kidneys. Under barbiturate anaesthesia (Sagital, May & Baker Ltd., UK), the left renal artery and vein were cannulated with a 1 inch 22-gauge Teflon catheter (Angiocath, Deseret Pharmaceutical Co. Inc., Utah, USA) and ligated proximally. Perfusion with PBS was commenced at 2 ml/min and continued for 10–15 min after venous effluent clearing. Specific or non-specific globulin was then perfused at 1 mg/min for 10 min. After further PBS perfusion for 20 min, cortex was taken for IF microscopy.

*Immunization of rabbits with Fx1A.* Six rabbits were injected 10 times 2-weekly with 30 mg rabbit Fx1A in CFA. Urinary protein excretion over 24 h was measured, rabbits killed 2 weeks following the final immunization, and their kidneys assessed by LM, IF and TEM.

*Preparation and use of kidney eluate from rabbits immunized with Fx1A; (Fx1A-Im eluate).* Pooled Fx1A immunized rabbit kidneys were homogenized, washed in PBS, and eluted twice in acid citrate buffer, pH 3·2 as previously described (Neale & Wilson, 1982a). Eluate was used by indirect IF to determine binding sites on normal rabbit kidney targets. Control eluate was prepared from the kidneys of normal rabbits and used at the same protein concentration as the Fx1A-Im eluate.

*Isoelectric focusing and immunoblotting of solubilised Fx1A.* Fx1A was extracted for 4 h at room temperature with either 1% deoxycholate (DOC) or PBS. Concentrated dialysed material (30 $\mu$l) was applied to separate isoelectric focusing gels (pH 3·5–10) under the following conditions. Prefocus for 30 min beginning at 12 mA then focused for 2 h until current 1–2 mA, on a Biorad

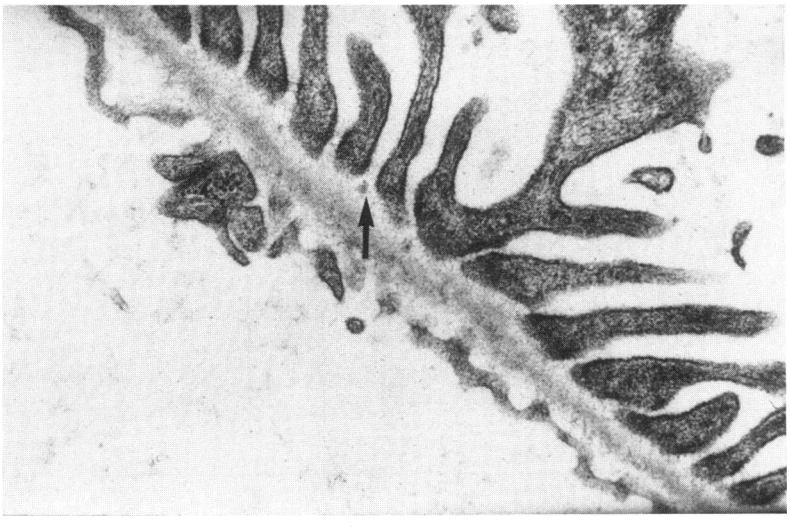

**Fig. 3.** A TEM photomicrograph of a rabbit GCW 12 h following IV sheep anti-rabbit Fx1A globulin. Arrow indicates subepithelial electron dense deposits. × 18,000.

**Table 1.** Specific binding of radiolabelled anti-rabbit Fx1A globulin. Specific binding of anti Fx1A is maximal in kidney at all time points studied. No radioactivity could be detected in samples of small intestine at the 7-day time point.

| | 24 hours (n = 3) | | | | 48 hours (n = 3) | | | | 7 days (n = 2) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | μg anti-Fx1A/gm tissue | ±s.d. | Total anti-Fx1A globulin organ bound (μg) | ±s.d. | μg anti-Fx1A/gm tissue | ±s.d. | Total anti-Fx1A globulin organ bound (μg) | ±s.d. | μg anti-Fx1A/gm tissue | ±s.d. | Total anti-Fx1A globulin organ bound (μg) | ±s.d. |
| Left kidney | 0·273 | 0·038 | | | 0·172 | 0·011 | | | 0·113 | 0·002 | | |
| Right kidney | 0·273 | 0·025 | | | 0·151 | 0·039 | | | 0·080 | 0·007 | | |
| Total kidney | | | 5·490 | 0·398 | | | 3·398 | 0·484 | | | 2·057 | 0·374 |
| Spleen | 0·730 | 0·096 | 0·957 | 0·095 | 0·410 | 0·031 | 0·654 | 0·288 | 0·064 | 0·017 | 0·100 | 0·041 |
| Liver | 0·083 | 0·021 | | | 0·052 | 0·028 | | | 0·074 | 0·018 | | |
| Small intestine | 0·012 | 0·002 | | | 0·007 | 0·004 | | | | | | |
| Lung | 0·109 | 0·039 | | | 0·079 | 0·026 | | | 0·045 | 0·041 | | |

electrophoresis cell using a Julabo F30 cooling unit and Biorad 3000/300 power pack. The focused samples were then transblotted to nitrocellulose (Schleicher and Schüll, Dassel, West Germany) using a Biorad Transblotting cell. Transferred proteins were exposed to sheep anti-rabbit Fx1A or rabbit anti-rabbit Fx1A antibody, each at 1:20 dilution; control sera from normal sheep and rabbits were also run in parallel. Any bound antibodies were revealed with peroxidase-labelled rabbit anti-sheep IgG (Dako) or goat anti-rabbit IgG (Miles-Yeda) at 1:1000 dilution, followed by chloronapthol (60 mg in 20 ml methanol and 100 ml tris buffered saline), and 30% $H_2O_2$.

## RESULTS

*The heterologous anti-rabbit Fx1A globulin model*

1 h post injection specific globulin bound in a confluent granular IF pattern along the GCWs (Fig. 1a) and was maximal at 24 h; C3 was similarly distributed. Autologous Ig deposition occurred after 4 days. LM revealed a mild proliferative GN at 48 h, with polymorphonuclear leucocytes (PMNs) only rarely evident, but maximal at 24 h; ($0{\cdot}8 \pm 0{\cdot}34$ PMNs per glomerular cross section). Sheep Ig could not be demonstrated in controls and LM revealed normal glomeruli. The onset of proteinuria was rapid (1 h), peaking at 48 h (2·6 times control) and declining thereafter (Fig. 2). Subepithelial electron-dense deposits (Fig. 3) were seen by 12 h, and at all time points thereafter in rabbits given specific globulin, but not in controls. Tubulo-interstitial structures showed no abnormality or Ig deposits in either the experimental or control rabbits.

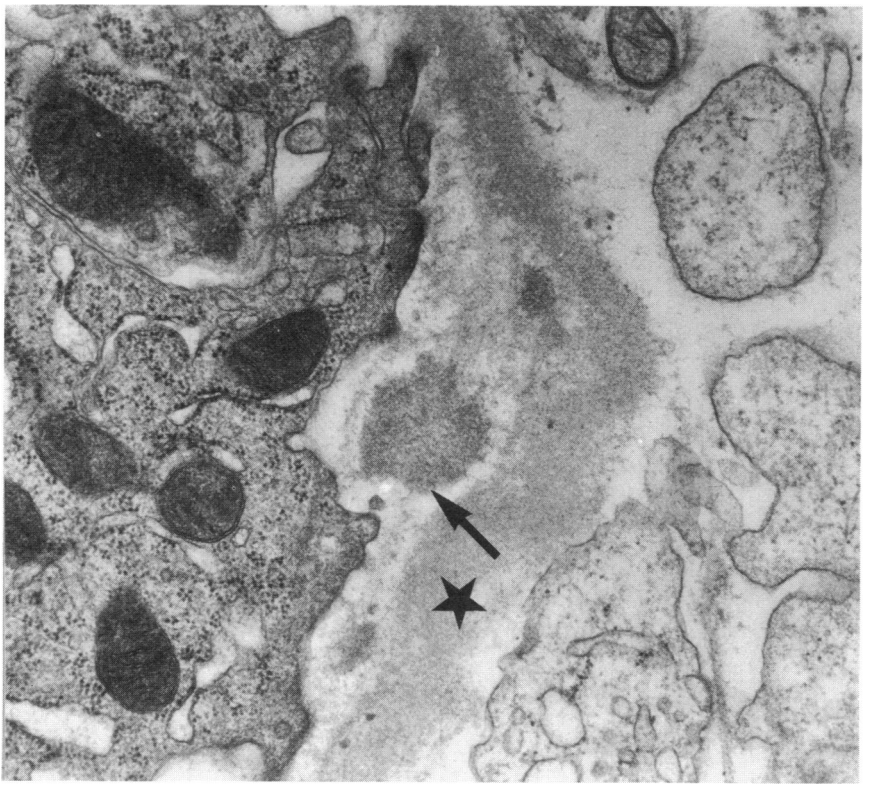

**Fig. 4.** A TEM photomicrograph of a tubulointerstitial area from a rabbit immunized repeatedly with rabbit Fx1A. The antiluminal border of a tubule (left) is separated from the TBM (★) by large electron dense deposits (→). ×27,000.

*The effects of leucopenia in rabbits given anti-rabbit Fx1A globulin*

Mustine maintained WCC at less than 1000 cells/mm$^3$ throughout the experiment, and only 0–2% PMNs at the time of globulin administration. Proteinuria in leucopenic rabbits given specific globulin was 2·7 times base-line, but did not develop in leucopenic rabbits given non-specific globulin. Sheep Ig was demonstrated in the glomeruli of rabbits given specific globulin and the pattern was identical to that in non-leucopenic rabbits.

PMN recruitment in the leucopenic rabbits was reduced compared to controls; no significant differences in total glomerular cell numbers were observed. Electron dense deposits occurred in the glomeruli of leucopenic rabbits given specific globulin but were not seen in controls. The nature and site of deposition was identical to that in unmanipulated rabbits given specific globulin.

*Specific binding of anti-rabbit Fx1A globulin*

Specific binding of anti-rabbit Fx1A globulin was greatest in the kidney and spleen at 24 h (Table 1), and fell steadily over the next 7 days. Binding in other tissues was insignificant.

*Perfusion of bloodless kidneys*

IF of sections from kidneys perfused with specific [but not non-specific] globulin revealed staining of GCWs (Fig. 1b). Appearances were identical to those *in vivo*. No staining of proximal tubules occurred in either group.

*Immunization with homologous Fx1A*

Immunized rabbits showed proteinuria [mean 56·0 ± 18·4 mg/24 h (range: 24–128)]. IF microscopy revealed granular Ig along the TBM. A variable peritubular interstitial infiltrate was observed. TEM indicated granular electron-dense deposits along the antiluminal border of the TBM and in the interstitial space at a sub-TBM site (Fig. 4).

*Indirect IF microscopy with Fx1A-Im and non-specific kidney eluates*

Fx1A-Im eluate stained normal rabbit proximal tubule brush border. There was a weak and inconsistent granular reactivity seen with normal rabbit GCW but no reactivity with TBMs or adjacent interstitial structures. Non-specific eluate showed no kidney reactivity. No intrinsic Fx1A-like antigens were evident at the bases of proximal tubules or subjacent areas.

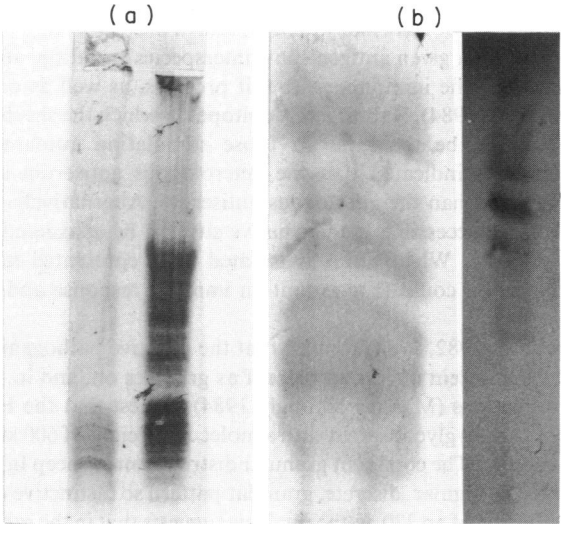


**Fig. 5.** (a) Isoelectric focusing gel of PBS (left) and DOC (right) solubilized rabbit Fx1A. (b) Immunoblots of heterologous (left) and autologous (right) anti-Fx1A on isoelectrically focused DOC-solubilized Fx1A.

*Iso-electric focusing and immunoblotting of extracted Fx1A*

Proteins extracted from Fx1A by DOC and by PBS and subjected to iso-electric focusing are shown in Fig. 5a. Immuno-blots of the nitrocellulose-transferred DOC-extracted proteins appear in Fig. 5b. There was some apparent homology between the two preparations but considerably more bands were revealed with the heterologous than with the homologous anti-Fx1A serum.

## DISCUSSION

From studies of certain well-defined models of GN, including Masugi nephritis, lectin nephritis, AHN, heterologous anti-Fx1A disease in rats, and spontaneous GN in rabbits, it is clear that immune reactions can occur *in situ* within the glomerulus. Antibody may bind to discrete, 'planted', or intrinsic GCW antigens rather than deposit from the circulation bound to antigen (Couser *et al.*, 1978; Golbus & Wilson, 1979; Madaio *et al.*, 1983; Neale *et al.*, 1982; Neale & Wilson, 1982a,b; Neale *et al.*, 1984; Van Damme *et al.*, 1978). In previous studies of immunologically mediated renal disease in rabbits following immunization with crude homologous kidney, the predominant lesion has been tubulointerstitial (Klassen *et al.*, 1971; Klassen *et al.*, 1977; Unanue *et al.*, 1966). Similarly, in the current study after immunization of rabbits with homologous Fx1A, a proteinuric tubulointerstitial lesion developed. Like observations have been made by other investigators using homologous rabbit kidney cortex (Klassen *et al.*, 1977; Unanue *et al.*, 1966).

Administration of heterologous anti-rabbit FX1A globulin to rabbits produced a proteinuric glomerular lesion, with some morphologic similarity to active and passive Heymann's nephritis in the rat, but with no evident tubular lesion. These observations imply that autoantibodies to Fx1A determinants do not recognize Fx1A or cross-reacting antigens if they are present in the glomerulus. The possibility that the antibody formed circulating complexes which were subsequently deposited in the glomerulus is unlikely, since it bound to glomeruli in perfused bloodless kidneys in the absence of circulating antigen. Also, by indirect IF the antibody reacted with normal glomeruli.

There are clearly differences between the active and passive anti-Fx1A diseases in the rabbit. Klassen *et al.* (1971), proposed that autologous or homologous antibody to cortical homogenate reacted with antigen which 'leaked' from proximal tubules forming interstitial immune complexes. The pooled sera from rabbits immunized with crude homologous kidney homogenate were shown to produce a similar tubular lesion in normal recipients, without glomerular damage; although in one, IgM was demonstrated along the GBM. However, the sera used had low or no anti-kidney antibody activity. In our study anti-Fx1A activity was high (1:64 by immunodiffusion). Antigen density and antibody concentration are likely to be critical in the production of glomerular damage.

Immunogenic epitopes on a given antigen show interspecies variation, and the host's response depends on the similarity of the immunogen to self proteins, as well as on immune regulatory mechanisms (Benjamin *et al.*, 1984). Rabbit Fx1A epitopes to which the sheep mounted an immune response were not likely to be the same as those stimulating autoantibodies. Indeed, the immunoblotting experiments indicated that the heterologous antiserum recognized more and different Fx1A determinants than the autologous antiserum. Alternatively, some Fx1A antigens could be immunologically inaccessible in their native site, or, be associated with MHC antigens restricting immune responses. When Fx1A is isolated and represented in a form not usually encountered *in vivo* the rabbit could then mount an immune response and peri-tubular damage ensue.

Kerjaschki & Farquhar (1982, 1983) showed that the putative pathogenic antigen responsible for AHN in rats is a glycoprotein of 330 kd present as granules on, and in, glomerular epithelial cells. More recent observations (Makker & Singh, 1984) suggest that the Heymann antigen is a glucose or mannose-containing glycoprotein with a molecular weight of 600 kd, of which the 330 kd molecule (gp 330) is a subunit. The confluent granular distribution of sheep Ig observed in our study is strikingly dissimilar to the regular, discrete, granular pattern so distinctive of passive Heymann's nephritis, and the distribution of gp 330, in the rat. This suggests that in the rabbit the distribution of the antigen (or multiple antigens) to which the heterologous antibody binds, is distinct from, or less anatomically restricted, than in the rat.

Ronco *et al.* (1984) produced a monoclonal antibody to rat Fx1A which bound a 90 kd protein localized to the proximal tubular brush border and the glomeruli. *In vivo* this antibody produced a transient proteinuric lesion with some immunohistological similarity to AHN. However, glomerular antibody binding by IF microscopy and by radiolabelled quantitative experiments was more closely analogous to that seen in our heterologous rabbit model.

Clearly, several GCW antigen-antibody systems could potentially produce glomerular injury in the rabbit by *in situ* mechanisms. Cornish *et al.* (1984) reported transient formation of fine subepithelial glomerular deposits in the rabbit using a guinea pig anti-'kidney tubular fraction 3' antibody. Electron microscopy indicated deposits close to slit pores. Only transient antibody deposition occurred. Unfortunately, no functional (measurement of proteinuria), or quantitative blinding studies were reported. The IF evidence of heterologous antibody deposition was more widespread and prominent in our model but there was dissociation between the IF and TEM appearances as electron dense deposits were small and almost exclusively subepithelial.

The temporal sequence in our heterologous anti-rabbit Fx1A model differs from that described in passive Heymann's nephritis. Also, the specific binding of radiolabelled globulin is at variance with that in the rat (Salant, Darby & Couser, 1980b). Binding of anti-rat Fx1A antibody in isolated glomeruli increased linearly from 4 h to 5 days, and proteinuria occurred at 4–5 days when total kidney bound antibody reached 200 $\mu$g. In contrast, proteinuria in the current study occurred rapidly, peaking at 48 h. Specific binding of antibody *in vivo* was maximal at the first time point studied (24 h) and decreased thereafter. The high specific binding to spleen was unexpected but was also reported by Ronco *et al.* (1984) with MoAb8. Observations by Bakker *et al.* (1979) where anti-rat T cell antibodies were present in nephrotoxic serum possibly account for this finding.

Passive HN in the rat is complement dependent: depletion of circulating PMNs has no effect on its development (Salent *et al.*, 1980a). The proteinuric glomerular lesion induced by anti-rabbit Fx1A globulin was also not influenced by leucopenia indicating that PMNs are not significant mediators in this model. The role of complement was not defined, although C3 was present within the glomeruli by direct IF microscopy.

In conclusion, our results indicate that anti-Fx1A disease in the rabbit and the rat can be distinguished not only by the autologous responses to Fx1A immunization but also by the nature and tempo of the lesions resulting from the administration of anti-Fx1A antibody. Immunoblotting results indicate that there are species differences in epitope recognition which, we speculate, may account for the dissociation between the heterologous and autologous models in the rabbit. The deposition of Ig and its IF pattern after perfusion of bloodless kidneys indicate an *in situ* immune mechanism is operative, rather than the deposition of previously formed circulating immune complexes.

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